1 **Engineering an *Escherichia coli* platform to synthesize designer biodiesels**

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11 **Running title:** Engineering designer biodiesels in *E. coli*

ABSTRACT

Biodiesels, fatty acid esters (FAEs), can be synthesized by condensation of fatty acid acyl CoAs and alcohols via a wax ester synthase in living cells. Biodiesels have advantageous characteristics over petrodiesels such as biodegradability, a higher flash point, and less emission. Controlling fatty acid and alcohol moieties are critical to produce designer biodiesels with desirable physiochemical properties (e.g., high cetane number, low kinematic viscosity, high oxidative stability, and low cloud point). Here, we developed a flexible framework to engineer *E. coli* cell factories to synthesize designer biodiesels directly from fermentable sugars. In this framework, we designed each FAE pathway as a biodiesel exchangeable production module consisting of acyl CoA, alcohol, and wax ester synthase submodules. By inserting the FAE modules in an engineered *E. coli* modular chassis cell, we generated *E. coli* cell factories to produce targeted biodiesels (e.g., fatty acid ethyl (FAEE) and isobutyl (FAIbE) esters) with tunable and controllable short-chain alcohol moieties. The engineered *E. coli* chassis carrying the FAIbE production module produced 54 mg/L FAIbEs with high specificity, accounting for >90% of the total synthesized FAEs and ~4.7 fold increase in FAIbE production compared to the wildtype. Fed-batch cultures further improved FAIbE production up to 165 mg/L. By mixing ethanol and isobutanol submodules, we demonstrated controllable production of mixed FAEEs and FAIbEs. We envision the developed framework offers a flexible, alternative route to engineer designer biodiesels with tunable and controllable properties using biomass-derived fermentable sugars.

Key words: fatty acid ethyl esters; fatty acid isobutyl esters; advanced biofuels; designer biodiesels; modular cell chassis; biodiesel modules.

1. INTRODUCTION

Microbial conversion of renewable and sustainable biomass feedstocks has emerged as a promising technology to produce alternative hydrocarbon biofuels such as higher alcohols, diesels, gasolines, and jet fuels (Somerville et al., 2010; Tilman et al., 2009). The challenge is to engineer novel microbial biocatalysts that can produce target biofuels at high yields, titers, and productivities from recalcitrant lignocellulosic biomass, and replace those synthesized from petroleum-based feedstocks in a competitive manner (Himmel et al., 2007; Stephanopoulos, 2007).

Fatty acid esters (FAEs), mono-alkyl esters, can be used as biodiesels and specialty chemicals for the flavoring, cosmetic and pharmaceutical industries (Gandhi, 1997). Biodiesels are an important class of renewable liquid transportation fuels. Since the last decade, the increasing biodiesel demand has resulted in a significant increase in biodiesel production from 9 million gallons in 2001 up to 1.3 billion gallons in 2013 (EIA, 2011). The advantages of using biodiesels over petrodiesels are that biodiesels are biodegradable, have a higher flash point, produce less emission, and can be produced from renewable and sustainable feedstocks (Demirbas, 2009). To function like petrodiesels, biodiesels are required to have desirable physiochemical properties (e.g., high cetane number, low kinematic viscosity, high oxidative stability, and low cloud point) to meet standards such as the American Society for Testing and Materials (ASTM) D6751 specification (Atabani et al., 2012; Knothe, 2008). These physiochemical properties are governed by carbon compositions of fatty acid and alcohol moieties. For instance, biodiesels derived from soybean in the United States, are primarily comprised of methyl oleate (73-92%), which is recommended for enrichment to enhance biodiesel properties (Knothe, 2008). In comparison to fatty acid methyl esters (FAMES), other

types of biodiesels, that have longer and branched carbon chains in fatty acid and/or alcohol moieties, can help increase the cetane number while lowering the kinematic viscosity, an important property for biodiesels to work at low temperatures (Knothe, 2005, 2008; Knothe et al., 2003; Knothe and Steidley, 2005).

At present, FAMES are primarily used as biodiesels, and are produced chemically by trans-esterification of triglycerides (e.g., vegetable oils and animal fats) and methanol in presence of a strong acid or base catalyst at elevated temperature (~60-110°C) (Yusuf et al., 2011). In contrast, enzymatic or microbial conversion is a promising alternative route that can produce biodiesels from renewable and sustainable feedstocks (Fjerbaek et al., 2009; Kalscheuer et al., 2006a; Steen et al., 2010). Certain plants and bacteria are capable of producing FAMES natively by using *S*-adenosyl-L-methionine (SAM)-dependent methyltransferases which condense fatty acids and SAM (Yang et al., 2006). A recombinant *E. coli* harnessing this enzyme was successfully demonstrated to produce FAMES albeit at a very low titer (~2.8 mg/L) (Nawabi et al., 2011). Fatty acid ethyl esters (FAEEs) are another type of biodiesels that can be produced by recombinant *E. coli* and *S. cerevisiae* (Kalscheuer et al., 2006a; Runguphan and Keasling, 2014; Shi et al., 2014; Steen et al., 2010; Yu et al., 2011). By engineering a dynamic sensor-regulator system in a recombinant *E. coli* to sense intracellular precursor acyl CoAs, FAEE production was improved up to 1.5 g/L, about 28% of the maximum theoretical yield (Zhang et al., 2012). Due to broad substrate specificity of the wax ester synthase AtfA from *Acinobacter baylyi* (Stoveken et al., 2005), a variety of FAEs other than FAMES and FAEEs can be synthesized (Guo et al., 2014; Kalscheuer et al., 2006b; Tao et al., 2015; Wierzbicki, 2013).

In this study, we developed a flexible framework to engineer an *E. coli* platform to synthesize designer biodiesels directly from fermentable sugars by using the modular pathway

engineering approach (Layton, 2014; Trinh, 2015; Tseng and Prather, 2012; Xu et al., 2013). Within this framework, we designed each FAE pathway as an exchangeable biodiesel production module consisting of acyl CoA, alcohol, and wax ester synthase submodules. By inserting the FAE modules in an engineered *E. coli* chassis cell, we developed microbial cell factories capable of producing targeted biodiesels in a plug-and-play fashion with enhanced production. By manipulating mixtures of alcohol submodules, we demonstrated the feasibility to engineer designer biodiesels with tunable and controllable short-chain alcohol moieties.

2. MATERIALS AND METHODS

2.1 Strain construction

Bacterial strains and plasmids of this study are presented in Table 1. *E. coli* TOP10 strain was used for molecular cloning while BL21 (λ DE3) was applied for protein expression and purification. For biodiesel production, *E. coli* MG1655 and its derivatives were characterized. Mutants with chromosomal gene deletion were constructed by P1 transduction (Trinh et al., 2006). A T7 polymerase gene was inserted into the chromosome of a mutant by using the prophage λ DE3 (cat#69734-3, EMD Millipore, Billerica, MA, USA). Table 2 shows a list of primers used to verify gene deletion or insertion in mutant chromosomes.

2.2 Plasmid/pathway construction

We designed each FAE pathway as an exchangeable biodiesel production module consisting of acyl CoA, alcohol, and wax ester synthase submodules (FIG. 1). Each submodule was organized in one operon that contained at least one gene under a constitutive T7 promoter.

The acyl CoA and wax ester synthase production submodules were organized in a pETite* vector backbone (Layton, 2014) using the ampicillin selection marker. The alcohol production submodule was organized in a different pETite* vector using the kanamycin selection marker. Table 2 presents a list of primers used for the plasmid construction and validation. All constructed plasmids were verified by PCR amplification, digestion, and sequencing.

Construction of the acyl CoA plus wax ester synthase submodule. The plasmid pCT69a– pETite* P_{T7}::RBS::tesA*::RBS::fadD*::RBS::atfA*::T_{T7} was designed for the acyl CoA plus wax ester synthase submodule that converts acyl ACPs to free fatty acids to acyl CoAs and then FAEs (FIG. 1). The designed operon consisted of a mutated thioesterase gene *tesA**, a mutated acyl CoA synthetase gene *fadD**, and a mutated wax ester synthase gene *atfA** under the T7 promoter. The gene *tesA** was derived from *tesA* whose leader peptide sequence was removed to keep the encoded protein TesA* cytosolically (Cho and Cronan, 1995). The genes *fadD** and *atfA** had the NdeI and BglIII restriction sites removed, respectively, to make them compatible with the BglBrick cloning technique (Anderson et al., 2010). The construction of pCT69a is described next.

First the genes *tesA* and *fadD* were amplified from the genomic DNA of *E. coli* MG1655 using the primers P064_f2/P064_r and P065_f2/P065_r, respectively. The NdeI site of *fadD* was removed by using the primers P066_f/P066_r via site-directed mutagenesis (cat #: 210519-5, Agilent Technologies, Santa Clara, CA, USA) to generate *fadD**. Both *tesA* and *fadD** were inserted into pETite* to create pCT42 via the BglBrick Gene Assembly method (Anderson et al., 2010). Next the gene *atfA* was amplified from the genomic DNA of *Acinetobacter sp. ADP1* using the primers P063_f/P063_r. The BglIII site of *atfA** was removed by using the primers P015_f/P015_r via site-directed mutagenesis. This modified *atfA** was inserted into pCT42 to

create pCT63k (kanamycin selection). The leader sequence of *tesA** was then removed from pCT63k to generate pCT69k by using the primers MW.24f/P064_r. The plasmid pCT69a (ampicillin selection) was constructed by the Gibson Gene Assembly method using 2 DNA pieces: a backbone fragment amplified from pCT69k with omission of the kanamycin gene by using the primers pk124_f/pk124_r and the ampicillin gene insert amplified from pUC19 (Lucigen Corp., Middleton, WI) with the primers pk125_f/pk125_r.

Construction of the ethanol production submodule. The ethanol production submodule (pCT24, pETite* P_{T7}::RBS::pdc::RBS::adhB::T_{T7}) was designed to convert pyruvate to ethanol and was previously engineered (Layton, 2014).

Construction of the isobutanol production submodule. The isobutanol submodule pCT13, pCOLA P_{T7}::RBS::ilvC::RBS::ilvD::RBS::alsS::T_{T7} – P_{T7}::RBS::kivd::RBS::adhE::T_{T7} was constructed previously to convert pyruvate to isobutanol (Trinh et al., 2011).

Construction of the FAEE production module. The FAEE module was assembled by using a two plasmid system pCT69a/pCT24. A single-plasmid FAEE module pCT77k was also created by the BglBrick Gene Assembly method from 2 DNA pieces: a backbone fragment from pCT24 digested from BamHI/XhoI and an insert fragment derived from pCT69a digested with BglII/XhoI. The plasmid pCT77a (ampicillin selection) was assembled by the Gibson Gene Assembly method using 2 DNA pieces: a backbone fragment amplified from pCT77k with omission of the kanamycin gene by using the primers pk124_f/pk124_r and the ampicillin gene insert amplified from pUC19 with the primers pk125_f/pk125_r.

Construction of the FAIbE module. The FAIbE module was assembled by using a two plasmid system pCT69a/pCT13.

2.3 Medium and culturing conditions

Medium. For molecular cloning, Lauria Bertani (LB) complex medium (pH7) plus appropriate antibiotics was used. For single plasmid selection, ampicillin and kanamycin were used at working concentrations of 100 µg/mL and 50 µg/mL, respectively. For double plasmid selection with two different antibiotics, the concentration of each antibiotic was reduced to half. For the strain characterization experiments, a hybrid M9 medium (pH7) was used including M9 salt (Sambrook, 2001) plus 1 mL/L of 1M MgSO₄, 100 µL/L of 1M CaCl₂, 1 mL/L of 1 mg/L thiamine HCl, 1 mL/L stock trace metals solution (Trinh et al., 2008), 20 g/L glucose, appropriate antibiotics, and 5 g/L of yeast extract.

Strain characterization. High-cell density batch experiments were conducted at 37°C and 175 rpm. Single colonies were picked and inoculated into 8 mL of LB medium in 15 mL tubes overnight. Each overnight seed culture was then transferred into a 250 mL flask containing 50 mL fresh hybrid M9 medium with an initial OD_{600nm}~0.05. When reaching OD_{600nm}~0.6, the culture was induced with IPTG at a working concentration of 0.5 mM until OD_{600nm}~1.5-2.0. The cells were centrifuged, washed, and resuspended into the fresh M9 hybrid medium in a 15-mL glass tube with 10 mL working volume and initial OD_{600nm}~5.0. After 30-minute incubation, cells were induced with IPTG at a working concentration of 0.5 mM. For exogenous alcohol addition experiments, the culture medium was also added with alcohols at a working concentration of 2 g/L. After 12 hr, cell cultures were collected for analysis.

Fed-batch experiments were conducted in 250 mL flasks with a working volume of 100 mL M9 hybrid medium for 72 hr. Cell cultures were maintained at pH7 by manual adjustment with 5 M NaOH every 24 hr throughout the course of experiments. Samples were collected every 24 hr immediately followed by fresh supplementation of 20 g/L glucose from a (50X) concentrated stock.

Each characterized strain was conducted at least in 3 independent experiments. Each value presented in the RESULTS was reported as mean $\pm$ 1 standard deviation.

2.4 Analytical methods

High pressure liquid chromatography (HPLC). The Shimadzu HPLC system equipped with both UV-VIS and RID detectors, and Aminex 87H column (BioRad Inc., Hercules, CA) was used to quantify extracellular metabolites such as sugars, alcohols, and organic acids. The HPLC column was run isocratically at 0.6 mL/min and 50°C with 10 mM H₂SO₄ as a mobile phase (Trinh et al., 2011).

Gas chromatography coupled with mass spectrometry (GC/MS). To quantify free fatty acids, cell cultures were first lysed with 6 M HCl and extracted with ethyl acetate containing 5 mg/L of pentadecanoic acid as an internal standard. The organic phase was collected and evaporated to dryness, and redissolved in 100 μ L of methanol. Esterification of free fatty acids was then carried out in methanol containing 2% H₂SO₄ at 80°C for 2 hr. The samples were cooled to room temperature, and 300 μ L of 0.9% sodium chloride was added thereafter. FAMES were extracted with 300 μ L of hexane and analyzed by GC/MS. To prepare samples for FAEE and FAIbE analysis, 100 μ L cell cultures were lysed with 6 M HCl and directly extracted with 1 mL ethyl acetate containing 1mg/L of pentadecanoic acid ethyl ester as an internal standard.

The GC/MS run was conducted by using the HP6890 GC/MS system equipped with a HP 5973 mass selective detector and a 30 m $\times$ 0.25 mm i.d., 0.25 μ m film thickness DB5-MS column (Phenomenex Inc., Torrance, CA) with an attached 10 m guard column. A 1 μ L portion of the extracted sample was injected into the DB5 column using splitless injection (1 μ L at 280°C). Helium was used as a carrier gas (1.1 μ L/min). The mass transfer line and ion source

were set at 250 °C and 200 °C, respectively. All the esters were detected with electron ionization in both scan mode (50 to 650 m/z) and selected ion monitoring mode at m/z 88 and parent ion of each compound (for quantitative analysis). The column temperature was initially held at 160 °C for 1 minute, then increased by 20 °C per min for 7 min, and held at 300 °C for 2 min.

2.5 Enzyme expression, isolation, and characterization

The gene *atfA* was cloned into pETite with a His-tagged sequence at C-terminus to create pCT16, expressed in *E. coli* BL21(λDE3) under a T7 promoter, purified, and characterized to verify its catalytic functions. For expression, the overnight cell culture BL21(λDE3) pCT16 was subcultured in a fresh LB medium (~pH7, 37 °C) containing 20 g/L glucose and 50 µg/L kanamycin. Upon reaching exponential growth ($OD_{600nm} \sim 0.5$), cells were induced with IPTG at a working concentration of 0.5 mM and incubated for 20 hr at 20 °C before being collected for protein purification.

To isolate AtfA, cell pellets were collected, washed twice, resuspended in phosphate buffered saline (PBS) solution, and disrupted by sonication. The crude cell extract was collected by centrifugation at 12,000xg and 4 °C for 20 min. His-tagged AtfA in the crude cell extract was semi-purified by immobilized Ni²⁺-affinity chromatography (cat# 30210, Qiagen Inc., Valencia, CA, USA) according to the manufacturer's instructions. The proteins were eluted with a linear gradient of imidazole (from 10 to 500 mM) in water. The pure fractions were concentrated with Amicon Ultra Centrifugal Filter Units (Merck Inc., Kenilworth, NJ) and the semi-purified proteins were analyzed by 12% SDS-PAGE.

For *in vitro* characterization of AtfA, each enzymatic assay was conducted in a 1.5 mL tube containing 50 mM PBS, 5 µM palmitoyl CoA, 4 mM short-chain alcohol, and 100 ng/µL

purified protein at pH7 and 37 °C for 90 min (Stoveken et al., 2005). Samples were extracted with equal volume of ethyl acetate containing 1 µg/mL ethyl pentadecanoate as an internal standard and analyzed by GC/MS.

3. RESULTS AND DISCUSSION

3.1 Characterization of the alcohol wax ester synthase AtfA for *in vitro* FAIbE production

The wax ester synthase AtfA is a very promiscuous enzyme (Stoveken et al., 2005). While the *in vivo* study suggests that AtfA has broad substrate specificity towards branched, short-chain alcohols (Kalscheuer et al., 2004; Wierzbicki, 2013), it has not yet been tested *in vitro* against these alcohols. Here we expressed the His-tagged AtfA in the soluble form, semi-purified, and characterized it with various short-chain alcohols. The result first confirmed the activity of AtfA towards ethanol, propanol, and butanol (positive controls) (FIG. 2) as demonstrated previously (Stoveken et al., 2005). In addition, AtfA successfully condensed palmitoyl CoA and isobutanol to produce isobutyl palmitate as well as exhibited catalytic function towards isopropanol to produce isopropyl palmitate. The substrate preference of AtfA towards short-chain alcohols can be ranked as follows: C2 ≤ iC3 < C3 < iC4 < C4 (FIG. 2). In other words, AtfA has higher activities for the linear than branched short-chain alcohols, given the same carbon chain length. Based on our *in vitro* study, AtfA can be deployed to construct the FAIbE-producing pathway in *E. coli*.

3.2 Establishing the FAIbE-producing pathway in *E. coli*

Wildtype *E. coli* does not produce FAIbEs endogenously. To engineer the FAIbE-producing pathway in *E. coli*, we first constructed the acyl CoA plus wax ester synthase production submodule, pCT69a, that can convert acyl ACPs to free fatty acids, to acyl CoAs, and then FAIbEs with exogenous addition of isobutanol (FIG. 1). We transformed pCT69a into EcCT001 to create EcCT101, and characterized it in high-cell density batch experiments for FAIbE production by exogenously adding 2 g/L isobutanol to the medium. The result shows that EcCT101 successfully produced a mixture of FAIbEs including 9.87 ± 1.12 mg/L isobutyl laurate (C12-iC4), 4.06 ± 0.49 mg/L isobutyl myristate (C14-iC4), and 0.54 ± 0.20 mg/L isobutyl palmitate (C16-iC4) with higher specificity towards C12 lauryl CoA after 12 hr (FIG. 3). We also observed the production of mixed FAEEs as byproducts including 0.52 ± 0.20 mg/L ethyl laurate (C12-C2), 0.27 ± 0.09 mg/L ethyl myristate (C14-C2), 0.48 ± 0.28 mg/L ethyl palmitate (C16-C2), and 0.30 ± 0.10 mg/L ethyl stearate (C18-C2). However, EcCT101 produced FAIbEs with higher selectivity, accounting for 90% of the total FAEs produced. FAEEs were produced as by-products because AtfA could condense acyl CoAs produced by pCT69a and ethanol produced by the endogenous ethanol fermentative pathway of *E. coli*. Taken all together, the result confirms that the acyl CoA plus wax ester synthase production submodule, pCT69a, worked as expected.

To demonstrate the endogenous FAIbE production directly from glucose, we next constructed the FAIbE production module (pCT69a/pCT13) by combining the acyl CoA plus wax ester synthase (pCT69a) and isobutanol (pCT13) production submodules, transformed it into EcCT001 to create EcCT201, and characterized the engineered strain in the high-cell density batch experiments. The result shows that EcCT201 successfully produced FAIbEs directly from glucose with a titer of 11.42 ± 1.23 mg/L after 12 hr including 64% C12-iC4, 22% C14-iC4, and

14% C16-iC4 (FIGS. 4B and S1). It was also observed that EcCT201 produced 1.39 ± 0.08 mg/L FAEEs as minor byproducts (~10% of the total FAEs, FIG. 4C) with the following distribution—55% C12-C2, 18% C14-C2, 19% C16-C2, and 9% C18-C2 (FIG. 4A).

Our results suggested that the alcohol precursors were not likely limiting during the FAEE/FAIbE biosynthesis because residual alcohols were detected at a reasonable level in the fermentation medium (FIG. 5A). Further, we observed that AtfA exhibited high specificity towards C12 acyl CoA. The C12 fatty acid moieties in FAIbEs/FAEEs were dominant whereas C12 fatty acid was only the minor composition of all residual free fatty acids produced (Fig. 5B). Ideally, the distribution of longer fatty acid moieties in FAEs is preferred to produce biodiesels with higher cetane numbers. Like the above alcohol exogenous addition experiments, EcCT201 produced FAIbEs with high selectivity up to 90% of the total generated FAEs and synthesized C12-iC4 as the major component of FAIbEs (FIGS. 4C and S1). This result was consistent with the *in vitro* characterization of AtfA that exhibited higher activities toward longer than shorter chain alcohols.

3.3 Engineering *E. coli* metabolism for enhanced endogenous FAIbE production

Effect of blocking the β -oxidation cycle on the endogenous FAIbE production. The β -oxidation cycle is a competitive pathway of the FAIbE biosynthesis for the precursor acyl CoAs. To block the β -oxidation cycle for enhancing the FAIbE production, we deleted the gene *fadE*, encoding the acyl CoA dehydrogenase that catalyzes the first step of the cycle (Campbell and Cronan Jr, 2002), from EcCT001 to create EcCT002. We then transformed the FAIbE production module into EcCT002 to create EcCT202 and characterized it in the high-cell density batch experiments. The result shows that EcCT202 achieved a 12% increase in the total FAE

production and produced 12.76 ± 1.75 mg/L FAIbEs and 1.21 ± 0.54 mg/L FAEEs with the same distribution of fatty acid moieties (FIGS. 4 and S1).

Effect of enhancing acyl CoA pools on the endogenous FAIbE production. In parallel to blocking the β -oxidation cycle, we targeted to direct more carbon flux to acyl CoA biosynthesis pathway. We deployed a modular *E. coli* chassis EcCT003 derived from TCS083 with additional *fadE* disruption. In brief, TCS083 was designed to disrupt all major fermentative pathways, oxidative pentose phosphate pathway, and inefficient electron transport system for enhanced biomass (Trinh et al., 2006) and ethanol (Trinh et al., 2008) production. We transformed the FAIbE production module into the modular chassis EcCT003 to create EcCT203 and characterized it in high-cell density batch experiments.

The result shows that EcCT203 achieved a significant 4.7 fold increase in total FAE production with 53.67 ± 6.82 mg/L FAIbEs (FIG. 4B) and 5.49 ± 0.70 mg/L FAEEs (FIG. 4A) after 12 hr. EcCT203 produced FAIbEs with high selectivity accounting for >90% of total FAEs (FIG. 4C) and had C14-iC4 as a major product (FIG. S1). The produced FAIbEs contained 29% C12-iC4, 54% C14-iC4, and 17% C16-iC4 while FAEEs consisted of 25% C12-C2, 45% C14-C2, 25% C16-C2, and 5% C18-C2. The shift in the fatty acid moieties of FAIbEs/FAEEs appears to be consistent with the availability of >C14 free fatty acids produced (FIG. 5B). Compared to EcCT201 and EcCT202, EcCT203 not only achieved much higher FAE production but also increased the longer carbon-chain lengths in fatty acid moieties that can improve the cetane number of biodiesels produced.

Increasing FAIbE production in the fed-batch operation. We further characterized EcCT203 in fed-batch experiments to examine kinetics of cell growth, FAE production, and glucose consumption (FIG. 6). The result shows that EcCT203 entered the stationary phase after

24 hr while still producing FAEs with high selectivity (>86%) of FAIbEs and with the distribution of fatty acid moieties similar to that investigated in the high-cell density experiments. However, the FAE production started to be reduced after 48 hr. After 72 hr, EcCT203 produced up to 187.76 ± 18.23 mg/L FAEs consisting of 88% FAIbEs and 12% FAEEs.

3.4 Engineering designer biodiesels by controlling alcohol moieties of FAEs

One other strategy to control physiochemical properties of designer biodiesels is to manipulate alcohol moieties in FAEs. With the modular pathway design strategy, we can easily mix and match submodules such as the acyl CoA plus wax ester synthase submodules (pCT69a) with ethanol (pCT24) and isobutanol (pCT13) submodules. To demonstrate this, we constructed pCT77a by combining pCT69a/pCT24, transformed it together with pCT13 into the modular chassis EcCT003 to create EcCT205, and characterized the engineered strain in high-cell density batch experiments. The mutant EcCT205 produced up to 45.12 ± 0.09 mg/L FAEs consisting of 76% FAIbEs (44% C12-iC4, 45% C14-iC4, and 11% C16-iC4) and 24% FAEEs (45% C12-C2, 39% C14-C2, 14% C16-C2 and 2% C18-C2) (FIG. 4). Compared to EcCT201, EcCT202, and EcCT203, EcCT205 increased the FAEE fraction from 3~9% up to 24% of the total FAEs. The result suggests that it is very feasible to control any desirable distribution of FAEs by titrating fluxes through ethanol and isobutanol submodules via manipulation of promoter strength (Cox et al., 2007), ribosome binding site (Anderson et al., 2010; Salis et al., 2009), plasmid copy number (Kittleson et al., 2011) and regulatory elements (Zhang et al., 2012).

3.5 Elucidating potential bottlenecks hindering biodiesel production

Accumulation of biodiesel precursors, ethanol and isobutanol, of our characterized strains in the fermentation broth suggests that the ethanol/isobutanol submodules were not likely limiting (FIG. 5A). It was likely the acyl CoA plus wax synthase submodules that limited production of designer biodiesels. Specifically, the low production of designer biodiesels might have been caused by the incompatible specificities of TesA, FadD and AtfA towards fatty acid moieties. *In vitro* enzymatic data extracted from the Bonner and Block's prior study (Bonner and Bloch, 1972) shows that the specificity and catalytic efficiency of TesA is ~2.2 times higher for a pool of C16:0, C18:0 and C20:0 acyl CoAs than for a pool of C12:0 and C14:0 acyl CoAs (FIG. S2A). This substrate specificity of TesA towards longer acyl CoAs is desirable for biosynthesis of designer biodiesels, and is consistent with the distribution of free fatty acids produced at relatively high levels in EcCT201, EcCT202, EcCT203, and EcCT205 (e.g., C16, C18, and C20 fatty acids representing 75-97% of the total free fatty acids produced) in our study (FIG. 5B).

In contrast to the substrate preference of TesA, the specificity and catalytic efficiency of FadD is ~6.8 times higher for a pool of C12:0 and C14:0 fatty acids than for a pool of C16:0, C18:0, and C20:0 fatty acids (FIG. S2B) (Kameda and Nunn, 1981). Indeed, we observed that this substrate specificity of FadD accounted for the dominant C12-C14 fatty acid moieties of biodiesels produced by EcCT201, EcCT202, EcCT203, and EcCT205. In addition to the incompatibility of substrate preferences of TesA and FadD, high specificity of AtfA towards palmitoyl CoA (Stoveken et al., 2005) might have limited the designer biodiesel production in our study.

Taken altogether, our results suggest that FadD is the rate limiting step for production of designer biodiesels. It is critical for future studies to address the incompatible substrate

preferences among TesA, FadD, and AtfA of the acyl CoA plus wax ester synthase submodules to enhance designer biodiesel production.

CONCLUSION

Biodiesels are a good source of renewable liquid transportation biofuels that can replace petrodiesels by harnessing renewable and sustainable feedstocks. Compared to the conventional biodiesel production from transesterification of plant oils or animal fats, the microbial conversion route is potentially more advantageous and flexible for directly manipulating fatty acid and alcohol moieties, bypassing the chemical transesterification steps, and utilizing a diversity of scalable feedstocks. In this study, we developed a flexible framework capable of engineering designer biodiesels with tunable and controllable properties from fermentable sugars by using the modular pathway engineering approach.

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TABLE LEGENDS

Table 1. Plasmids and strains used in this study

Table 2. Primers used for plasmid and strain construction and validation

Table 1. Plasmids and strains used in this study

Strain/plasmid	Genotype	Source
Plasmids		
pETite C-His	pBR322 <i>ori</i> ; kan ⁺	Lucigen
pCT13	pCOLA P _{T7} ::RBS:: <i>alsS</i> ::RBS:: <i>ilvC</i> ::RBS:: <i>ilvD</i> ::T _{T7} -P _{T7} ::RBS:: <i>kivd</i> :: RBS:: <i>adhE</i> ::T _{T7} -kan ⁺	Trinh 2011
pCT16	pETite C-His p _{T7} ::RBS:: <i>atfA</i> ::T _{T7} -kan ⁺	this study
pCT24	pETite* pT7:: RBS:: <i>pdC</i> :: RBS:: <i>adhB</i> ::T _{T7} -kan ⁺	Layton 2014
pCT42	pETite P _{T7} ::RBS:: <i>tesA</i> ::RBS:: <i>fadD</i> *::T _{T7} -kan ⁺	this Study
pCT63k	pETite P _{T7} ::RBS:: <i>tesA</i> ::RBS:: <i>fadD</i> *::RBS:: <i>atfA</i> *::T _{T7} -kan ⁺	this Study
pCT69k	pETite P _{T7} ::RBS:: <i>tesA</i> *::RBS:: <i>fadD</i> *::RBS:: <i>atfA</i> *::T _{T7} -kan ⁺	this Study
pCT69a	pETite P _{T7} ::RBS:: <i>tesA</i> *::RBS:: <i>fadD</i> *::RBS:: <i>atfA</i> *::T _{T7} -amp ⁺	this study
pCT77k	pETite* pT7:: RBS:: <i>pdC</i> ::RBS:: <i>adhB</i> ::T _{T7} - P _{T7} ::RBS:: <i>tesA</i> * ::RBS:: <i>fadD</i> *::RBS:: <i>atfA</i> *::T _{T7} -kan ⁺	this study
pCT77a	pETite* pT7:: RBS:: <i>pdC</i> ::RBS:: <i>adhB</i> ::T _{T7} -P _{T7} ::RBS:: <i>tesA</i> *:: RBS:: <i>fadD</i> *::RBS:: <i>atfA</i> *::T _{T7} -amp ⁺	this study
Strains		
<i>Acinetobacter</i> <i>sp. ADPI</i>	wildtype	ATCC 33305
TOP10	F ⁻ <i>mcrA</i> Δ (<i>mrr-hsdRMS-mcrBC</i>) ϕ 80 <i>lacZ</i> AM15 Δ <i>lacX74</i> <i>nupG</i> <i>recA1</i> <i>araD139</i> Δ (<i>ara-leu</i>)7697 <i>galE15</i> <i>galK16</i> <i>rpsL</i> (Str ^R) <i>endA1</i> λ ⁻	Invitrogen
BL21 (DE3)	F ⁻ <i>ompT</i> <i>hsdS_B</i> (<i>r_B⁻ m_B⁻) <i>gal dcm</i> (λDE3)</i>	Invitrogen
JW5020-1	BW25113 Δ <i>fadE</i> ::kan ⁺	CGSC#11134
MG1655	F ⁻ λ ⁻	ATCC 47076
TCS083	MG1655, Δ <i>zwf</i> :: <i>Andh</i> :: <i>AsfC</i> A:: <i>AmaeB</i> :: <i>AldhA</i> :: <i>AfrdA</i> :: <i>ApoxB</i> :: <i>Apta</i> ::kan ⁻ (cured)	Trinh 2008
EcCT001	MG1655 (λ DE3)	this study
EcCT002	EcCT001 Δ <i>fadE</i> ::kan ⁻	this study
EcCT003	TCS083 (λ DE3) Δ <i>fadE</i> ::kan ⁻	this study
EcCT101	EcCT001 pCT69a; amp ⁺	this study
EcCT201	EcCT001 pCT69a/pCT13; kan ⁺ and amp ⁺	this study
EcCT202	EcCT002 pCT69a/pCT13; kan ⁺ and amp ⁺	this study
EcCT203	EcCT003 pCT69a/pCT13; kan ⁺ and amp ⁺	this study
EcCT205	EcCT003 pCT77a/pCT13; kan ⁺ and amp ⁺	this study

501 **Table 2.** Primers used for plasmid and strain construction and validation
502

Primers	Primer Sequence
<i>Primers used to build plasmids</i>	
P015_f	5'-CTTTATTCAAGACCTGGTGAATGATATCC-3'
P015_r	5'-GGATATCATTACACCAGGTCTTGAATAAAG-3'
P063_f	5'-AAAAAAGAATTCATGAGATCTAAGGAGATATAATGCGCCCATTACAT CCGATTG-3'
P063_r	5'-AAAAAACTCGAGTTAGGATCCTTAATTGGCTGTTTTAATATC-3'
P064_f2	5'-AAAAAAGAATTCATGAGATCTAAGGAGATATAATGCGCCCATTACAT CCGATTG-3'
P064_r	5'-AAAAAACTCGAGTTAGGATCCTTATGAGTCATGATTTACTAAAG-3'
P065_f2	5'-AAAAAAGAATTCATGAGATCTAAGGAGATATAATGTTGAAGAAGGTT TGGCTTAAC-3'
P065_r	5'-AAAAAACTCGAGTTAGGATCCTCAGGCTTTATTGTCCACTTTG-3'
P066_f	5'-GTTAACCCATACGATATTGATTATC-3'
P066_r	5'-GATAATCAATATCGTATGGGTAAAC-3'
pk124f	5'-GTTTTTTCCTGTTTGGTCATTTACCAATGCTTAATCAGTG-3'
pk124r	5'-GAGATTTTCAGGAGCTATGAGTATTCAACATTTCCGTGTC-3'
pk125f	5'-GACACGGAAATGTTGAATACTCATAGCTCCTGAAAATCTC-3'
pk125r	5'-CACTGATTAAGCATTGGTAAATGACCAAACAGGAAAAAAC-3'
mw.24f	5'-AAAAAACATATGGCGGACACGTTATTGATTCT-3'
<i>Primers used for verifying gene deletion and addition</i>	
P043_KO_ldhA_f	5'-TTTCTGGCGGATTTTTATCG-3'
P043_KO_ldhA_r	5'-CGTCAACGGCACAAGAATAA-3'
P051_KO_frdA_f	5'-AGTTGATGCAACCGGAGAAC-3'
P051_KO_frdA_r	5'-ACGGCGAGACAAATTTTACG-3'
maeA_fwd_KO	5'-CAGCGTAGTAAATAACCCAACC-3'
maeA_rev_KO	5'-GACAGCTTAACGGCTTTGTAG-3'
P044_KO_zwf_f	5'-CGATGAACGGTCGAAGTTTT-3'
P044_KO_zwf_r	5'-TGCCATAGCAGCAATACTCG-3'
P049_KO_ndh_f	5'-GCAGACGCACAAATTCAAGA-3'
P049_KO_ndh_r	5'-ACGGGAACACCTCCTTCTTT-3'
maeB_fwd_KO	5'-GATGATAATGGCGAATGGAC-3'
maeB_rev_KO	5'-CGTTCTTTATCCATGAGTCG-3'
P045_KO_pta_f	5'-TCACTGGTGGTATCGGTGAA-3'
P045_KO_pta_r	5'-GAATGCGAAATGAGTGTGGA -3'
P046_KO_poxB_f	5'-ATGGATATCGTCGGGTTTGA-3'
P046_KO_poxB_r	5'-AAGCAATAACGTTCCGGTTG-3'
fadE_fwd_KO	5'-CGCATTATTCGGCCTACGGTTC-3'
fadE_rev_KO	5'-CCAGACTCCGTTTGTAAATGCAACAC-3'
DE3_fwd	5'-ATGAACACGATTAACATCGC-3'
DE3_rev	5'-TTACGCGAACGCGAAGTC-3'

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FIGURE LEGENDS

FIG. 1: Biosynthetic pathway for endogenous production of FAEEs and FAIbEs from biomass-derived fermentable sugars in *E. coli*.

FIG. 2: Relative *in vitro* enzymatic activities of AtfA normalized with the one using ethanol and palmitoyl CoA as substrates. Each data point is an average $\pm$ 1 standard deviation.

FIG. 3: GC/MS chromatogram of FAIbE production by EcCT101 after 12 hr in high-cell density batch experiments with exogenous addition of 2 g/L isobutanol to the medium.

FIG. 4: Characterization of EcCT201 (EcCT001 pCT69a/pCT13), EcCT202 (EcCT002 pCT69a/pCT13), EcCT203 (EcCT003 pCT69a/pCT13), and EcCT205 (EcCT003 pCT69a/pCT77k) for FAE production after 12 hr in high cell-density batch experiments. **A.** FAEE production. **B.** FAIbE production. **C.** Distribution of FAEEs and FAIbEs.

FIG. 5: **(A)** Residual alcohols and **(B)** free fatty acids (FAs) detected after 12 hr in high cell-density batch experiments of EcCT201, EcCT202, EcCT203, and EcCT205.

FIG. 6: Endogenous production of FAEs by EcCT203 in fed-batch experiments. **A.** Kinetics of cell growth and glucose consumption. **B.** Kinetics of FAEE and FAIbE production. It should be noted that throughout the experiment the pH was manually adjusted to 7 from ~5.4-5.6 every 24 hr right after sampling. Abbreviation: DCW: dry cell weight.

FIG. 1

534 **FIG. 2**

FIG. 3

FIG. 4

FIG. 6